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Ecdysteroid Production in *Drosophila melanogaster* Reared on Defined Diets

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Larvae of *Drosophila melanogaster* were reared aseptically on defined diets containing either cholesterol, campesterol or sitosterol as the only dietary sterol. Sterol analyses of pupae revealed that insects reared on campesterol and sitosterol diets contained 3.3 and 8.1% cholesterol, indicative of an ability to accumulate this sterol. Ecdysone and 20-hydroxyecdysone were the predominant ecdysteroids in insects from all diet studies, though makisterone A was detected in pupae reared on campesterol and sitosterol.

Drosophila Dietary sterols Dealkylation Ecdysteroids

INTRODUCTION

An insect's need for a dietary source of sterol was first established in the blow fly *Lucilia sericata* by Hobson (1935), and this dietary requirement for sterol is now generally acknowledged for all insects. Most phytophagous insects can convert 28- and 29-carbon plant sterols to cholesterol via dealkylation, while other species cannot (Svoboda and Thompson, 1985). In Diptera, sterol metabolism differs markedly between species. Larvae of the mosquito *Aedes aegypti* are able to dealkylate plant sterols to cholesterol (Svoboda *et al.*, 1982), while larvae and adults of the house fly *Musca domestica* are incapable of this conversion (Kaplanis *et al.*, 1963, 1965). In the genus *Drosophila*, Kircher and coworkers (1984) reported that several cactophilic species could not convert plant sterols to cholesterol, though Redfern (1984) concluded *D. melanogaster* could dealkylate a yeast sterol (ergosterol) to cholesterol. The inability of *D. melanogaster* to either dealkylate sitosterol or convert desmosterol to cholesterol was subsequently demonstrated using radiolabeled sterols in a defined diet (Svoboda *et al.*, 1989).

Dietary sterols serve as the precursors for the steroid molting hormones, and insects have been shown to produce a variety of C_{27}, C_{28} and C_{29} ecdysteroids, though one type usually predominates in any given species (Feldlaufer, 1989; Grieneisen, 1994). However, whole body extracts of *D. melanogaster* larvae and pupae have been shown to contain mixtures of C_{27} and C_{28} ecdysteroids, as did extirpated brain-ring gland complexes (Redfern, 1984; Pak and Gilbert, 1987). Redfern (1986) extended his *in vitro* studies and reported that the production of a C_{28} molting hormone by brain-ring gland complexes was associated with certain levels of the C_{28} sterol campesterol in the larval diet.

In our current study, we report the sterol and ecdysteroid composition of *D. melanogaster* pupae, derived from larvae that were reared aseptically on defined diets containing either C_{27}, C_{28}, or C_{29} sterols.

MATERIALS AND METHODS

Insects and diets

Flies from colonies of the Oregon R strain of *Drosophila melanogaster* were cultured axenically on an agar/casein-based defined medium described by Sang (1978), with the following modifications. Choline chloride was substituted for lecithin, and to minimize the amount of endogenous sterol in test media, the casein was extracted six times with chloroform–methanol (2:1). After purification by HPLC and recrystallization, test sterols (cholesterol, campesterol and sitosterol) were coated on the dry components of the diet with methylene chloride to achieve a final sterol concentration of 0.03% (wet weight basis). Eggs were surface-sterilized with 0.1% sodium hypochlorite and transferred aseptically into flasks containing diet. All flasks were incubated at 25°C, and pupae collected from each test diet were weighed and stored in methanol at −15°C until analyzed.

Chemicals

All solvents for extraction and purification were reagent grade, redistilled. Solvents for HPLC were

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obtained from Burdick & Jackson. Ecdysone, 20-hydroxyecdysone and makisterone A were purchased from Simes (Milano, Italy). Makisterone C (24-ethyl-20-hydroxyecdysone) was a gift of D. H. S. Horn (Melbourne, Australia).

Extraction and purification

Pupae from each sterol diet were homogenized in methanol (2×) and 75% methanol–water (1×). Filtrates were combined and dried *in vacuo*. Individual residues were partitioned between 70% methanol–water and *n*-hexane (countersaturated) and each phase was back-washed with the appropriate solvent. Pooled hexane phases were dried and used to isolate and identify neutral sterols, while the pooled methanolic residues were used for ecdysteroid analyses. A portion of test diet, prior to the incorporation of test sterols, was also analyzed for sterol composition.

Sterol analyses

Methodologies used to analyze and identify sterols are described by Svoboda and Lusby (1986). Briefly, the crude lipids in the hexane residue were saponified (4% potassium hydroxide in methanol) and the sterols in the nonsaponifiables were purified by column chromatography on acid-grade alumina and neutral-grade alumina in ether–hexane systems. Sterol fractions from all samples were quantitatively and qualitatively analyzed by capillary GLC and identifications were confirmed by GLC–mass spectrometry.

Ecdysteroid analyses

Methanolic residues remaining from the hexane partition were further partitioned between *n*-butanol and water (countersaturated). The water phase was back-washed with fresh butanol and the pooled butanol phases were combined and dried. Residues were fractionated on silica Sep-Paks®, eluted with 5-ml portions of ethanol in chloroform (5, 15, 25, 40%) and stripped with ethanol. Ecdysteroids were present in the 15 and 25% fractions (determined by RIA), and these fractions were combined and eluted from a C_{18} Sep-Pak in a methanol–water system previously described (Thompson *et al.*, 1985).

HPLC-RIA

An LDC solvent delivery system (Thermo Separation Products) was used in conjunction with a Waters 990 photodiode array detector monitoring the eluant at 220 and 248 nm. Putative pupal ecdysteroids from the Sep-Pak fractionations were injected in methanol onto an IBM C_8 column (150 mm × 4.6 mm; 5 μm particle size) eluted with 35% methanol–water at 1 ml/min. Column temperature was held constant at 33°C. One-milliliter fractions were collected for RIA.

The "H-22" antiserum used in this study was a gift of L. I. Gilbert (Chapel Hill, North Carolina) and was raised in rabbits injected with a hemisuccinate derivative of ecdysone, conjugated at C-22 (Warren and Gilbert, 1986). [23,24-^{3}H]Ecdysone (spec. act. 83 Ci/mmol; Dupont) was used as the labeled ligand. All assays were performed in triplicate as previously described (Feldlaufer *et al.*, 1984). The cross-reactivity factors for 20-hydroxyecdysone (3.84), makisterone A (2.88) and makisterone C (1.60) were determined in our laboratory by comparing the mass of each ecdysteroid required to displace 50% of the radiolabel, relative to the mass of ecdysone required. While ecdysone was used to generate the standard curve, all values reported in the results are corrected for cross-reactivity.

RESULTS

Dietary and pupal sterols

Despite solvent extraction of the casein prior to inclusion in the diet, 0.0005% cholesterol and less than 0.0001% campesterol and sitosterol could be detected in unsupplemented diet. Since test sterols were added to yield a final concentration of 0.03%, a 60–300-fold excess of test sterol over any contaminating sterol existed. The predominant sterol in all pupae was highly reflective of the particular dietary sterol available to larvae, accounting for over 90% of the total tissue sterol (Table 1). It is noteworthy, though, that flies reared on the campesterol diet contained 3.3% cholesterol, and those reared on the sitosterol-based diet contained 8.1% cholesterol.

Pupal ecdysteroids

Qualitative and quantitative analyses of ecdysteroids in *D. melanogaster* pupae reared on defined diets are presented in Table 2. As expected, the major ecdysteroids present in pupae reared on a cholesterol-based diet were ecdysone (8.8 ng/g) and 20-hydroxyecdysone (21.2 ng/g), which accounted for all of the ecdysteroids detected. Together, these C_{27} compounds also accounted for the majority of ecdysteroids detected in flies reared on campesterol (64%) and sitosterol (79.3%) diets. The C_{28} ecdysteroid makisterone A was detected in flies reared on campesterol (11.5 ng/g) and sitosterol (1.8 ng/g), though the 29-carbon ecdysteroid makisterone C was not detected in any of the samples.

TABLE 1. Comparison of *D. melanogaster pupal* sterols from insects reared on diets containing different sterols

	Dietary sterol		
Pupal sterols	Cholesterol	Campesterol	Sitosterol
Cholesterol	691.2* (96.0)†	30.5 (3.3)	55.1 (8.1)
Campesterol	8.6 (1.2)	877.8 (95.0)	12.2 (1.8)
Sitosterol	18.0 (2.5)	14.8 (1.6)	612.7 (90.1)
Total	717.8 (99.7)	923.1 (99.9)	680.0 (100)

Dietary sterols were added to achieve a final concentration of 0.03% (30 mg) on a wet weight basis. Unsupplemented diet was shown to contain 5 μg/g fresh weight cholesterol and < 1 μg/g fresh weight campesterol and sitosterol.

*μg/g fresh weight.

†Relative per cent of total sterol.

TABLE 2. Ecdysteroid composition of *D. melanogaster* pupae reared on specific dietary sterols

	Ecdysteroid (ng/g fresh weight)		
Dietary sterol	20-Hydroxyecdysone	Ecdysone	Makisterone A
Cholesterol	21.2	8.8	0
Campesterol	16.1	4.4	11.5
Sitosterol	4.8	2.1	1.8

Ecdysteroids were quantified by RIA after HPLC, and values were corrected for cross-reactivity.

DISCUSSION

Sterol utilization and molting hormone composition in *D. melanogaster* reared on sterol-defined diets is qualitatively similar to that reported in the house fly, *M. domestica* (Feldlaufer and Svoboda, 1991). In both species, the C_{27} steroids ecdysone and 20-hydroxyecdysone were the predominant ecdysteroids detected, regardless of the larval dietary sterol. Both species of flies also produced lesser amounts of the C_{28} ecdysteroid makisterone A on campesterol-based diets. This compound was also detected in *D. melanogaster* reared on sitosterol, but not in house flies.

House flies have been shown to selectively accumulate cholesterol from their diets (Robbins, 1963; Feldlaufer and Svoboda, 1991). The relative amounts of cholesterol detected in *D. melanogaster* pupae reared on campesterol and sitosterol diets indicate that this species, likewise, may possess a mechanism for sequestering cholesterol. This was particularly evident in sitosterol-fed flies, where cholesterol accounted for 8.1% of the total pupal sterol though only trace amounts (0.0005%) were present in the diet (as a contaminant). We calculated (based on a pupal weight of 1.5 mg per individual) that each individual would have to consume about 16.5 mg of diet over the course of its development in order to accumulate the 55.1 μg (8.1%) of cholesterol found. Preferential uptake of specific sterols has been demonstrated in other species of *Drosophila*. Kircher *et al.* (1984) reported that two species of cactophilic flies, *D. mojavensis* and *D. nigrospiracula*, selectively absorbed cholesterol and campesterol from artificial diets, and discriminated against the uptake of sitosterol from their host cacti. These findings in cactophilic flies are somewhat contrary to reports involving *D. melanogaster* where, based on body weight and developmental time, sitosterol appeared to support growth and development somewhat better than cholesterol (Cooke and Sang, 1970; Svoboda *et al.*, 1989). It is interesting to note that total molting hormone production in our study differed markedly between those flies reared on sitosterol and those reared on either cholesterol or campesterol. Insects reared on cholesterol and campesterol diets produced a total of 30.0 and 32.0 ng of molting hormone per gram fresh weight, respectively. Pupae from sitosterol-fed larvae, however, contained only 8.7 ng/g of molting hormone, in spite of the fact they contained more cholesterol than the campesterol-reared insects. Whether the cholesterol in sitosterol-fed flies was needed to meet other physiological and/or structural requirements or was otherwise unavailable for ecdysteroid biosynthesis is a matter of speculation. It is also difficult to say whether these results are any indication of substrate suitability.

Svoboda *et al.* (1989) demonstrated that *D. melanogaster* could not dealkylate [^{14}C]sitosterol nor convert [^{14}C]desmosterol to cholesterol. Although other radiolabeled sterols like campesterol were not included in the study, their conclusion that *D. melanogaster* was incapable of dealkylating other C_{28} and C_{29} phytosterols was based on the fact that desmosterol has been shown to be a common intermediate in the pathway for dealkylation and conversion to cholesterol of all phytosterols examined to date. (Ikekawa, 1985; Svoboda and Thompson, 1985; Rees, 1989; Grieneisen, 1994). Our present study is consistent with this view, since in all instances, the predominant dietary sterol (either cholesterol, campesterol or sitosterol) accounted for more than 90% of the total sterol isolated from *D. melanogaster* pupae. While C_{27} ecdysteroids were the major ecdysteroids detected in all insects in our study, we believe this to be due to the ability of *D. melanogaster* larvae to selectively accumulate cholesterol, regardless of the amount in the diet. The report that *D. melanogaster* could dealkylate the C_{28} sterol ergosterol was based on the detection of C_{27} molting hormone (ecdysone) in culture medium (Redfern, 1984). Given both the likelihood that artificial diets contain some endogenous sterol, and that *D. melanogaster* larvae can sequester even minute amounts of cholesterol, it may be interesting to further investigate ergosterol metabolism in greater detail.

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